Recombinant gene Medicine of Adenovirus Vector and P53 gene for Treating Proliferative Diseases

Field of the Invention

The present invention relates to genetic engineering technology, especially to the genetic recombinant medicine that is made of the recombinant sequences of human tumor suppressor gene p53 and adenovirus vector for treatment of proliferative diseases.

Background of the Invention

Proliferative disease is a genic disease featured by cell hyperplasia and/or abnormal expression of metabolite products. It is a benign hyperplasia, which could cause dysfunctions in all human tissues and organs such as skin, marrow and mammary gland. The scar is the inevitable outcome of the human wound recovering process. Any kind of wound recovering will result in scars. There are two kinds of scars including normal scar and pathological scar. Pathological scar includes hyperplastic scar and cheloid, which both cause neoplastic proliferation and dysfunction. It is one of the four most difficult problems in medical science. Cheloid is a skin hyperplastic disease. It is over hyperplasia caused by voluntarily or involuntarily induced abnormal collagen accumulation that usually occurs in skin injury. Its clinical symptoms include over development, over the original border of the injury, invasion of the neighbor tissues, non-recession, and reoccurrence after surgery. It has been proved by many experiments that p53 gene mutation occurs in cheloid formation. However, there is still no effective and specific treatment for cheloid in nowadays. It is also one of the most important problems in orthopaedic surgery.

The common medicines for proliferative disease are (1) corticosteroid, which is effect to small pathological scar, but could induce local skin shrink, pigment decrease or decolor, telangiectasis, even skin necrosis and elkosis. It will seriously cause general reaction, such as hypertension, osteoporosis, digestive tresis, teratocarcinoma, even Cushing's response; (2) tretinoin, which is seldom used for treating scar; (3) tranilast, which need to administrate for more than 6 months. Surgical treatment, laser treatment, radiation treatment and compression

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method also can be used for treating proliferative disease.

In the developing of the study of the relative genes in pathological scar, some controlling genes of fibroblast cells propagation-apoptosis and metabolism of collagen have been cloned and described. Therefore, gene therapy for proliferative disease appears.

The present applicant discloses a recombinant, which can amplify and propagate in specific genetic-engineered cell lines, and also can express tumor suppressor protein in eukaryote cells. The recombinant vector can be either DNA virus or RNA virus. The preferred vector is adenovirus vector or combined vector containing adenovirus vector sequence. The most preferable vector is the adenovirus vector.

The human tumor suppressor gene can be any tumor suppressor genes, the most preferable one is p53.

The recombinant combined with adenovirus vector and p53 gene is defined as recombinant p53 adenovirus, which has the following sequence:

the right end of adenovirus

ATGTTTACCGCCACACTCGCAGGGTCTGCACCTGGTGCGGGTCTCATCGTA
CCTCAGCACCTTCCAGATC$_{70}$TCTGACATGCGATGTCGACTCGACTGCTTCGC
GATGTACGGGCCAGATATACGCGTATCTGAGGGGACTAGGGTGTGTTTAGGC
GAAAAGCGGGGCTTCGGTTGTACGCGGTTAGGAGTCCCCTCAGGATATAGTA
GTTTCGCTTTTGCATAGGGAGGGGGAAATGTAGTCTTATGCAATACTCTTGTA
GTCTTGCAACATGGTAACGATGAGTTAGCAACATGCCTTACAAGGAGAGAAA
AAGCACCGTGCATGCCGATTGGTGGAAGTAAGGTGGTACGATCGTGCCTTAT
TAGGAAGGCAACAGACGGGTCTGACATGGATTGGACGAACCACTGAATTCC
GCATTGCAGAGATATTGTATTTAAGTGCCTAGCTCGATACAATAAACGCCATTT
GACCATTCACCACATTGGTGTGCACCTCCAAGCTTGGTACCGAGCTCGGATC
CCG$_{523}$CTAGAGCCACCGTCCAGGGAGCAGGTAGCTGCTGGGCTCCGGGGA
CACTTTGCGTTCGGGCTGGGAGCGTCTTTCCACGACGGTGACACGCTTCCC
TGGATTGGCAGCCAGACTGCTTTCCGGGTCACTGCC$_{655}$ATGGAGGAGCCGC
AGTCAGATCCTAGCGTCGAGCCCCCTCTGAGTCAGGAAACATTTTCAGACCT
ATGGAAACTACTTCCTGAAAACAACGTTCTGTCCCCCTTGCCGTCCCAAGCA

ATGGATGATTTGATGCTGTCCCCGGACGATATTGAACAATGGTTCACTGA
AGACCCAGGTCCAGATGAAGCTCCCAGAATGCCAGAGGCTGCTCCCCCCGT
GGCCCCTGCACCAGCAGCTCCTACACCGGCGGCCCCTGCACCAGCCCCCT
CCTGGCCCCTGTCATCTTCTGTCCCTTCCCAGAAAACCTACCAGGGCAGCTA
CGGTTTCCGTCTGGGCTTCTTGCATTCTGGGACAGCCAAGTCTGTGACTTGC
ACGTACTCCCCTGCCCTCAACAAGATGTTTTGCCAACTGGCCAAGACCTGCC
CTGTGCAGCTGTGGGTTGATTCCACACCCCCGCCCGGCACCCGCGTCCGC
GCCATGGCCATCTACAAGCAGTCACAGCACATGACGGAGGTTGTGAGGCGC
TGCCCCCACCATGAGCGCTGCTCAGATAGCGATGGTCTGGCCCCTCCTCAG
CATCTTATCCGAGTGGAAGGAAATTTGCGTGTGGAGTATTTGGATGACAGAAA
CACTTTTCGACATAGTGTGGTGGTGCCCTATGAGCCGCCTGAGGTTGGCTCT
GACTGTACCACCATCCACTACAACTACATGTGTAACAGTTCCTGCATGGGCG
GCATGAACCGGAGGCCCATCCTCACCATCATCACACTGGAAGACTCCAGTG
GTAATCTACTGGGACGGAACAGCTTTGAGGTGCGTGTTTGTGCCTGTCCTG
GGAGAGACCGGCGCACAGAGGAAGAGAATCTCCGCAAGAAAGGGGAGCCT
CACCACGAGCTGCCCCCAGGGAGCACTAAGCGAGCACTGCCCAACAACAC
CAGCTCCTCTCCCCAGCCAAAGAAGAAACCACTGGATGGAGAATATTTCACC
CTTCAGATCCGTGGGCGTGAGCGCTTCGAGATGTTCCGAGAGCTGAATGAG
GCCTTGGAACTCAAGGATGCCCAGGCTGGGAAGGAGCCAGGGGGGAGCAG
GGCTCACTCCAGCCACCTGAAGTCCAAAAAGGGTCAGTCTACCTCCCGCCA
TAAAAAACTCATGTTCAAGACAGAAGGGCCTGACTCAGACTGA$_{1837}$CATTCTC
CACTTCTTGTTCCCCACTGACAGCCTCCCACCCCCATCTCTCCCTCCCCTGC
CATTTTGGGTTTTGGGTCTTTGAACCCTTGCTTGCAATAGGTGTGCGTCAGA
AGCACCCAGGACTTCCATTTGCTTTGTCCCGGGGCTCCACTGAACAAGTTG
GCCTGCACTGGTGTTTTGTTGTGGGGAGGAGGATGGGGAGTAGGACATACC
AGCTTAGATTTTAAGGTTTTTACTGTGAGGGATGTTTGGGAGATGTAAGAAAT
GTTCTTGCAGTTAAGGGTTAGTTTACAATCAGCCACATTCTAGGTAGGGGCCA
CTTCACCGTACTAACCAGGGAAGCTGTCCCTCACTGTTGAATTTTCTCTAACT
TCAAGGCCCATATCTGTGAAATGCTGGATTTGCCCTACCTCGGAATGCTGGC
ATTTGCACCTACCTCACAGAGTGCATTGTGAGGGTT$_{2297}$AATGAAATAATGTAC
ATCTGGCCTTGAAACCACCTTTTATTACATGGGGTCTAGCGGGATCCACTAGT
AACGCCGCCAGTGTGCTGGAATTCTGCAGATATCCATCACACTGGCGGCCG
CTCGAGCATGCATCTAGAGCTCGCTGATCAGCCTCGACTGTGCCTTCTAGTT
GCCAGCCATCTGTTGTTTGCCCCTCCCCCGTGCCTTCCTTGACCCTGGAAG

GTGCCACTCCCACTGTCCTTTCCTAATAAAATGAGGAAATTGCATCGCAT
TGTCTGAGTAGGTGTCATTCTATTCTGGGGGGTGGGGTGGGGCAGGACAGC
AAGGGGGAGGATTGGGAAGACAATAGCAGGCATGCTGGGGATGCGGTGGG
CTCTATGGCTTCTGAGGCGGAAAGAACCAGCTGGGGCTCGAGGGGGATCC
CCACGCTAGAGCT$_{2733}$GACTATAATAATAAAACGCCAACTTTGACCCGGAACG
CGGAAAACACCTGAGAAAAACACCTGGGCGAGTCTCCACGTAAACGGTCAA
AGTCCCCGCGGCCCTAGACAAATATTA $_{2848}$- the left end of adenovirus 5

In which:

1. the right end of adenovirus 5 and the left end of adenovirus 5end are described in adenovirus 5 gene full sequence (Genbank No: NC_001406)
2. 1-70: the right arm of adenovirus (the 70th base locates at adenovirus gene sequence 3328)
3. 71-523 Rous Sarcoma Virus (RSV) LTR (promoter)
4. 524-655: 5' end non-translating region
5. 656-1837: p53 gene coding sequence
6. 1838-2733: 3' end non-translating region (poly Adenosine tail starting at 2298)
7. 2734-2848: the left arm of adenovirus (base at 2734 is positioned at 452 of adenovirus 5 gene sequence).

The expression cassette of this recombination is a specific sequence composed of promoter-p53cDNA-poly Adenosine. Its upstream sequence contains any eukaryotic cell promoter, prokaryotic cell promoter or virus promoter. Its downstream sequence contains any poly adenosine (polyA) of eukaryotic cells.

The recombinant DNA of this invention was obtained as described below. The recombinant virus vector was obtained in prokaryotic cells by homologous recombination. First, the recombinant product pGT-2 was constructed in *E.coli* by homologous recombination of the adenovirus with plasma pGT-1 which included the adenovirus's inverted repeat sequences on both ends. Second, recombinant product pGT-3 was constructed in *E.coli* by homologous recombination of pGT-2 with the artificial sequence "adenovirus right arm/promoter-p53cDNA-poly adenosine/adenovirus left arm". Finally, recombinant p53 adenovirus was

obtained by discarding the prokaryotic plasmid sequence using endonuclease *Pac*I. .

According to the above methods, the long terminal repeats (LTR) on both sides of adenovirus were amplified by PCR and *Pac*I restriction enzymatic sites were introduced respectively. Both LTR fragments were cloned into pUC18 vector and produced pGT-1 recombinant sequence. The constructed pGT-1 vector and adenovirus 5 gene sequence were then co-transfected the *E.coli* (BJ5183, preserved by SiBiono Company, preserve no.: P-e012). The adenovirus 5 gene sequence was then homologously combined with pGT-1. The positive virus clone was then amplified, screened by PCR, and tested using restriction enzymes. Finally, recombination vector pGT-2 which contains adenovirus 5 full gene sequence was obtained.

Using 5′ ATGGAGGAGCCGCAGTCAGATC and 5′ ATATCTGCAGAATTCCAGC AC as a primer, human tumor suppressor p53 gene was amplified by PCR. The full sequence of p53 gene (including the 5' and 3' non-translation sequence) was then cloned into vector pUC19 and tested by DNA sequencing. Next, the RSV (rous sarcoma virus) LTR sequence and the PA sequence of BGH and E1 sequence of adenovirus were amplified by PCR. A linker sequence was attached on one side of each aforesaid sequence and confirmed by DNA sequencing. In the next PCR reaction, LTR and PA sequences were attached to the 5' and 3' ends of p53, respectively. The adenovirus E1 section and its upstream sequence were combined to the outer side of p53, and the p53 gene expression cassette was thus constructed (Figure 1).

The *E.coli* BJ5183 was co-transfected by recombinant vector pGT-2 and p53 gene expression cassette, in which the homologous recombination occurred. The positive clones were then amplified, PCR screened, and tested via restriction enzymes digestion. The resulting recombinant vector was pGT-3, which contained most of the adenovirus 5 sequence (its E1 section and part of the upstream sequence were substituted by p53 expression cassette). The recombinant vector pGT-3 was linearized by *Pac*I and the sequence which originated from pUC18 was discarded. Then the pGT-3 was used to transfect 293 cells –(preserved by SiBiono Corp., preservation No.: E-393). The recombinant was packed in cells

and produced human tumor suppressor gene p53 cassette containing adenovirus cis-acting sequence and LTR promoter. The resulting recombinant p53 adenovirus vector had a high transfection rate, was easy to operate and was controlled by a single promoter.

This recombinant p53 adenovirus had the following characteristics:

It was constructed with adenovirus vector and p53 gene artificial expression cassette,

1. Structure: It was a living recombinant adenovirus which was different from other chemical synthetic medicines, herbs and genetically engineered medicines. It was highly biologically active, could be directly expressed *in vivo* and was highly effective in clinical application. Adenovirus could carry large gene fragment and had high transfection rate, which could be made as high titer virus particles and had a very broad host range, proved very safe. Its antigenicity was greatly reduced especially after being reconstructed. Thus the target gene was easily stabilized and expressed *in vivo*. In p53 artificial gene expression cassette, the expression of p53 gene was directly controlled by the single promoter of adenovirus vector, and the poly adenosine tail signal was added, thus an intact expression cassette was constituted.

2. Application: This recombinant p53 adenovirus was a broad spectrum anti-tumor medicine. It could be used to treat many malignant tumors. The phase II clinical trials indicated that it had significant treatment effects on head and neck squamous carcinomas and lung cancer, among others. The recombinant p53 adenovirus was especially effective in preventing tumor recurrence. The phase I clinical trial and 3 years post-surgery observations indicated that this recombinant p53 adenovirus had prevented the post-surgery relapse of the larynx cancer patients as a cancer vaccine.

The recombinant p53 adenovirus of this invention could be made into medicines for treatment of many malignant tumors in the experiement. And it could be made into medicines for prevention of tumorigenesis and post-surgery relapses of tumors.

The present applicant also found the recombinant p53 adenovirus~~it~~ could induce the abnormal hyperplastic cells expressing normal P53 protein, thus effectively d~~r~~epressing the cell reproduction and curing hyperplastic diseases including cheloid.

Summary of the Invention

The object of this invention is to recombine the potentially therapeutic genes with their vectors, thus providing a recombinant DNA of adenoviral vector and p53 gene for treatment of hyperplasia. This recombinant product will then induce the hyperplastic cells express normal P53 proteins. In this way the proliferation of the abnormal cells could be effectively repressed and could be used to treat hyperplastic diseases such as cheloid.

The recombinant medicine of this invention, was firstly transfected the specifically genetically engineered cells. Then the cells were grown, concentrated and purified into recombinant p53 adenovirus anti-hyperplasia injection which could be used in clinical application.

The 293 cells used in this invention (ATCC CRL-1573, 32^{th} generation, bought from ATCC, June 13^{th}, 1997) was screened from human embryonic kidney epithelial cells which was transformed by Adenovirus 5 (Ad5) DNA and contained 11% of the gene (including E1a) from Ad5 5' end. The cells were highly permissive for infection by adenovirus, and also permissive for growth of adenovirus.

The main contribution of this invention lies in taking advantage of the human tumor suppressor gene p53 which could suppress the growth of many abnormal hyperplastic cells. The suppressor gene was cloned into $E1^-$ adenovirus and was locally injected into body. Adenovirus help p53 gene enter the hyperplastic tissues. The expressed p53 protein could inhibit growth of the abnormal hyperplastic cells or even kill the abnormal cells. This invention also provided a method to prepare this recombinant p53 adenovirus product, which solved the problem caused by the instability of P53 protein (half-life = 20 minutes) *in vitro*.

The recombinant p53 adenovirus carried the human tumor suppressor gene p53 and expressed it directly in the abnormal hyperplastic cells, thus solved the

problem that recombinant genetically engineered product could not be made *in vitro* because instability of p53 protein. Using the adenovirus for treatment of hyperplastic diseases, P53 protein could be expressed *in vivo* continuously and highly efficiently. Furthermore, protein molecular modifications *in vivo* including phosphorylation, folding, and polymerization were equal to those in eukaryotic cells. The recombinant p53 adenovirus of this invention could be used to introduce the expression of p53 gene in eukaryotic cells by directly introducing the gene to the hyperplastic tissue to express protein, effectively using the patient as a source for producing human tumor suppressor factor P53 protein. This method successfully introduced the foreign p53 gene into the human body and allowed it to highly express in the hyperplastic tissue. This has made gene therapy of hyperplastic diseases possible.

Detailed Description of the Figures

Figure 1 is the schematic process of the construction of the recombinant medicine

Figure 2 is the flow chart of the experimental protocols for the production of the recombinant medicine

Figure 3 is the stability testing diagram of agarose gel electrophoresis of the recombinant gene after generations of passage, which was made by PCR to amplify the recombinant p53 adenovirus using 5′ CCACGACGGTGACACGCTTC and 5′ CAAGCAAGGGTTCAAAGAC as primer, and p53cDNA as template. PCR amplification of the recombinant p53 adenovirus obtains a 1400bp DNA fragment. 1. DNA marker; 2, 3, 4 The PCR results of the p53 cDNA

Figure 4 is the result analysis diagram of agarose gel electrophoresis of the PCR amplification of virus DNA, which was obtained 36 hours after cell 293 was infected by recombinant gene (preserved by SiBiono Corp., preservation No.: No-1, same as the following). The DNA fragment is 2750 bp.
1. DNA marker; 2. The PCR results of the recombinant p53 adenovirus
Figure 5 is the Western blot analysis result 36 hours after the Hep-2 and H1299 cellswere infected by recombinant adenovirus. The expression of p53 carried by the recombinant p53 adenovirus in Hep-2 cells and H1299 cells.

1. Protein marker; 2-3. Negative controls: Hep-2 cells and H1299 cells without infecting bySBN-1, respectively; 4-5. Hep-2 cells and H1299 cells infecting bySBN-1, respectively.

Figure 6 is the primary cultured fibroblasts of human hypertrophic scar *in vitro*. The continuous fibroblasts are in order, exhibit nodular or helix form. The fibroblasts are spindle or irregularity, and the cell boundary is clear under the optical microscope.

Figure 7 is the characterization of the fibroblast cells using S-P staining and vacuum. The cytolymph of the c cells using S-P staining and vacuum is brown, and the nucleuses are blue. All the continuous cells are fibroblasts and can produce protocollagen III because they are position cells.

Figure 8 is the microscopic photo of the killing effect of the recombinant gene to the scar fibroblast cells *in vitro*. B, C, D counts the configuration changes of the Scar fibroblast cells, which are infected with recombinant adenovirus after 24h, 48h, and 72h. The volume of the cells is increase, and change from spindle to polygonal, the cytolymph is also increase, and the nuclear division is decrease and appears dissociation and avalanche. However, the configuration of the control cells is not change significantly.

Figure 9 is the electron micrograph of the killing effect of the recombinant gene to the scar fibroblast cells *in vitro*. The observation (Fig. 9A, B, C) under transmission electron microscope exhibits the process of bubbling, appearing apoptotic body, and the apoptotic body casting in the cells with the recombinant medicine (MOI=200). Fig. 9D expresses another situation of apoptosis, which is the obvious increasing of chondriosomes.

Figure 10 includes the pictures of the effect of recombinant gene to the cheloid patient before and after treatment. The size of the scar had significantly decreased after gene therapy for 4 weeks.

Detail Description of Embodiments

The following embodiments are further descriptions for this invention. The practice

of this invention is not limited to these embodiments.

Experiment 1:

Construction and testing of the recombinant p53 adenovirus, as described in Figure 1 and 2.

1. Two primers were devised according to the published full sequence of p53 cDNA:

The two primers were 5′ ATGGAGGAGCCGCAGTCAGATC and 5′ ATATCTGCA GAATTCCAGCAC. Linker sequence was attached to both ends. Human p53 gene was PCR amplified using HeLa cell cDNA as a template. The experimental conditions were as follows:

For the first cycle, the DNA was denatured for 4 minutes at 94°C, annealing for 1 minute at 58°C, and then extended for 2 minutes at 72°C. For each of the rest of the cycles, the DNA was denatured for 1 minute at 94°C, annealing for 1 minute at 58°C, and then extended for 2 minutes at 72°C. There were 30 cycles total. Thus a large amount of p53 gene fragments were obtained. The p53 gene was then tested using agarose gel electrophoresis. The full sequence of p53 gene was recycled from the gel, purified, cut by restriction enzyme and inserted into pUC19 vector which was cut by the same enzyme. The fragment was then sequenced. The base sequence of the expressing section tested the same as the predicted amino acid sequence (concurrent with GenBank Acc XM_058834). Finally the fragment was cleaved by restriction enzyme and recollected.

2. LTR and PA sequences were PCR amplified. Their primers were respectively:

5′TCTGACATGCGATGTCGACTCG ,

5′ CGGCAGTGACCCGGAAAGCAG;

5′ TCACAGAGTGCA TTGTGAGGG,

5′ GCTCTAGCGTGGGGATCCC.

Linker sequences were attached to 5' primer and 3' primer.

LTR and PA were PCR amplified under the sameannealing conditionsdescribed above. The amplified fragments were purified and tested by sequencing.

3. Adenovirus E1 sequence was PCR amplified separately under the same PCR

conditions described above. Enzyme restriction sites for *Bam* HI and *Eco* RI were respectively linked to primers on both ends. The fragments were tested after being amplified.

4. The fragment from step 1 and the two fragments from step 2 were linked by PCR reaction respectively. The experimental conditions were as described above. PCR replication product LTR-p53-PA was obtained. The resulting sequence was tested by sequencing.

5. The fragment from step 3 and LTR-p53-PA from step 4 were linked by T4 DNA ligase. The resulting sequence was p53 gene cassette.

6. Inverted terminal repeat (IRT) sequences from both ends of adenovirus were PCR amplified under the same experimental conditions as described above. After being tested by sequencing, the resulting fragments were cloned to pUC18 vector. Thus the recombinant vector pGT-1 was obtained.

7. *E. coli* BJ5183 was co-transfected with recombinant vector pGT-1 and wild type adenovirus 5 (ATCC-VR-5, adenovirus 75, titer: 10(6.75) TCID (50)/ml) DNA. After being kept at 4°C for 1 minute, the transfected bacteria were then heat shocked at 42°C for 50 seconds, incubated at 4°C for 1 minute, then combined with 1ml LB media and incubated for 1 hour. The engineered bacteria were then spread on agar medium containing ampicillin and incubated for 24 hours. Single cells were picked by aseptic toothpick and put into a bottle with LB media, culture for 24 hours. Plasmid was extracted by common methods and screened by *Pac*I. The positive clones were pGT-2 (containing the full sequence of Ad5).

8. *E. coli* BJ5183 was co-transfected by recombinant vector pGT-2 and p53 gene cassette. The growing condition, screening and characterization methods were described above. Positive clones were pGT-3, which contained the adenovirus full gene sequence and inserted p53 expression cassette. The vector sequence originated from pUC18 was discarded after the clones were linearized by *Pac*I.

9. The positive linear plasmid were purified by CsCl and then used to transfect 293 cells using $CaCl_2$. Cells were collected after 7 days. The cells were centrifuged at 1000rpm for 15 minutes. The supernatant was discarded. Cells

were lysed 3 times at 37°C-80°C. It was again centrifuged at 4000rpm for 30 minutes. Precipitates were discarded. The supernatant was infected again to amplify the virus, and lysed the same way as described above. The resulting supernatant was density gradient centrifuged with CsCl, 60000rpm at 4°C for 16 hours. The band of recombinant adenovirus was extracted by No. 7 needle. The DNA fragment was added with N1H buffer and dialyzed at 4°C for 4 hours in a Spectra MW6000 dialysis bag. The DNA solution was sterilized by passing through a 0.25μm filter. Then the DNA solution were packed and stored at -80°C. Part of the resulting product was used in plaque assay and virus titer test.

10. Structure stability test for the recombinant adenovirus. The virus genomic DNA was obtained after generations of reproduction. The DNA fragments were PCR amplified using primers from both ends of p53 which were 5′CCACGACGGTGACACGCTTC and 5′ CAAGCAAGGGT TCAAAGAC. The results of agarose gel electrophoresis are shown in figure 3. Adenovirus arms at both sides of recombinant adenovirus were devised as primers: 5' TTT CTC AGG TGT TTT CCG C and 5' CAT CGT ACC TCA GCA CCT TC.. The results of PCR were shown in figure 4. The results above indicated that the structure of the recombinant p53 adenovirus was stable after many generations of reproduction.

11. The p53 gene expression test in Hep-2 and H1299 cells. 36 hours after being transfected by recombinant adenovirus, the Hep-2 and H1299 cells were lysed by common methods. The result of the western blot using P53 protein specific antibody was shown in figure 5.

Experiment 2:

The killing effect of recombinant adenovirus to fibroblast cells:

The culture of scar fibroblast cells *in vitro* (see figure 6): The Scar skin was obtained from surgery and cut into small pieces with size of 0.5-1cm^3 for each in aseptic conditions. The skin pieces were then immediately put into culture solutions with 1000U/ml ampicillin and streptomycin. The tissues were washed twice with PBS (with ampicillin and streptomycin) to remove fat and connective tissues. Then the tissues were washed again several times with D-Hank solution

until oil-free and clear. Next, the skin pieces were cut again until the size of $1mm^3$ for each piece. A few drops of serum were applied on the tissues and the pieces were spread out on the wall of the culture bottle. The bottle was then turned until the side with the tissues facing upwards and DMEM culture solution with 10% FBS was added (note: the tissues and the solution were separate). Then the bottle was sat in oven with 37°C and 5% CO_2 with the side of tissues facing upwards. 6-8hr later, the bottle was turned gently. The bottle was then sat for 3-4 days. The solution was changed every 3-4 days afterwards. New cells grew around the tissue pieces in 10 days and cell clusters came into being. The cells covered the whole bottle in about one month, forming single layer cells.

Cell types characterization using S-P staining and vacuum method

Sample preparations: The cells were digested using trypsin and made into cell suspension with concentration of 10000 cells/ml. Cover slips of 18×18mm were put in media dishes with size of 55mm. Two drops of cell suspension were put on each cover slip. And the cover slips were sat in oven with CO_2 for 6-8hrs and then washed thoroughly with PBS for 3 times. Next, the cover slips were put in pure acetone, fixed in room temperature for 15 min, and washed with PBS for 3 times. Immunochemical cell staining using S-P in vacuum (see figure 7): The cover slips were sat with the side of cells facing upwards on top of the slides. 50μl of mouse anti-human were applied on each slide. Same amount of PBS was added to the negative control group. The slides were put in vacuum oven with 66.7kPa for 10min and washed again with PBS for 3 times. Each slide was applied with 50μl of streptomycin biotin protein-peroxidase solution, sat in vacuum oven for 10min, washed with PBS for 3 times, re-stained with haematoxylin for 1min, applied with 1% alcohol for a few seconds, dehydrated with alcohol, applied with dimethylbenzene, sealed with gum and finally observed under microscope.

Observation of cell changes in microscope (see figure 8): $5×10^5$ counts of Scar fibroblast cells were inoculated in a $25cm^2$ bottle, and cultured for 24 hours. Then the culture solution was changed and the cells were infected with recombinant adenovirus of 200MOI. The configuration changes of cells were observed under microscope after 24h, 48h, and 72h.

The change of cells structure in electronic microscope (see figure 9): 5×10^5 counts of scar fibroblast cells were inoculated in a $25cm^2$ bottle, and cultured for 24 hours. Then the culture solution was changed and the cells were infected with recombinant adenovirus of 200MOI for 48h. The cells were digested by trypsin and the cells suspension was collected in agarose tube. The tubes were then centrifuged in 2000r/min for 15min to form the cells clump. The cells were then fixed with 2% of glutaraldehyde and 1% of osmium tetroxide. The cell clumps wrapped in agar were then dehydrated, permeated and wrapped with epoxy resin, thin-sliced, stained, observed and photoed under transmission electron microscope.

Experiment 3:

The treatment results of this recombinant adenovirus to cheloid in clinical research of scar gene therapy.

As shown in figure 10A and 10B, a female cheloid patient had scars cut off from her left chest after acnes. However, neoplastic scar was regenerated after the surgery. The local regeneration occurred in 3 years. Steroid and other common treatments showed no effect. The volume of the scar on her chest is $2\times1\times1cm^3$ (see figure 10A). The size of the scar had significantly decreased after gene therapy for 4 weeks. No other obvious side-effect showed except for self-limited fever. The recombinant adenovirus treatment for cheloid had been proved safe by clinical experiments.